

letters to nature

vehicle-injected or PTH-injected CD45.2⁺ C57BL/6 mice. Cells were injected in recipient B6.SJL mice lethally irradiated 24 h previously with 10 Gy of radiation. The relative contribution of engraftment from the different cell sources was assessed by flow cytometry of BM-MNCs using anti-CD45.1 and anti-CD45.2 antibodies (Pharmingen).

To assess post-transplantation PTH effects, lethally irradiated recipient C57BL/6 mice were injected with 2×10^5 BM-MNCs from a donor B6.SJL mouse. Twenty-four hours later, mice were injected with PTH or vehicle for four weeks.

Statistical analysis

Results are expressed as mean \pm s.e.m. Data were analysed using the unpaired two-tailed Student's *t*-test as appropriate for the data set. $P < 0.05$ was considered significant.

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Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours

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Activation of the Hedgehog (Hh) signalling pathway by sporadic mutations or in familial conditions such as Gorlin's syndrome is associated with tumorigenesis in skin, the cerebellum and skeletal muscle^{1,2}. Here we show that a wide range of digestive tract tumours, including most of those originating in the oesophagus, stomach, biliary tract and pancreas, but not in the colon, display increased Hh pathway activity, which is suppressible by cyclopamine, a Hh pathway antagonist. Cyclopamine also suppresses cell growth *in vitro* and causes durable regression of xenograft tumours *in vivo*. Unlike in Gorlin's syndrome tumours, pathway activity and cell growth in these digestive tract tumours are driven by endogenous expression of Hh ligands, as indicated by the presence of *Sonic hedgehog* and *Indian hedgehog* transcripts, by the pathway- and growth-inhibitory activity of a Hh-neutralizing antibody, and by the dramatic growth-stimulatory activity of exogenously added Hh ligand. Our results identify a group of common lethal malignancies in which Hh pathway activity, essential for tumour growth, is activated not by mutation but by ligand expression.

The Hh signalling pathway specifies patterns of cell growth and differentiation in a wide variety of embryonic tissues. Mutational activation of the Hh pathway, whether sporadic or in Gorlin's syndrome, is associated with tumorigenesis in a small subset of these tissues, predominantly skin, the cerebellum and skeletal muscle^{1,2}. Mutations that activate the Hh pathway include those that impair the ability of the transporter-like Hh receptor³ Patched (PTCH), the target of Gorlin's syndrome mutations) to restrain Smoothened (SMO)-mediated activation of transcriptional targets through the Gli family of latent transcription factors^{1,2,4,5}. Paradoxically, Hh pathway activity is associated with increased expression of *PTCH*, which is a transcriptional target of the pathway but is unable to restrain SMO when bound by Hh protein. Pathway activation, whether triggered by Hh binding or by *PTCH* mutation, requires SMO, a seven-transmembrane protein that binds to and is inactivated by the pathway antagonist cyclopamine⁶.

The recent finding that Hh pathway activity is important for growth of a significant proportion of small-cell lung cancers⁷, a tumour type not associated with Gorlin's syndrome, suggested that other, non-Gorlin's tumours might require Hh pathway activity for growth. We investigate here the role of pathway activity in tumours derived from the gut, a tissue with prominent and diverse roles for Hh signalling in developmental patterning, and in mature tissue homeostasis. A role in homeostasis is suggested by the expression of Hh ligands and target genes in postnatal gut epithelium and mesenchyme^{8–11} (Fig. 1a).

We began our examination of gut-derived tumours by testing for expression of *Sonic hedgehog* (SHH) and *Indian hedgehog* (IHH), which encode members of the Hh ligand family that are expressed in

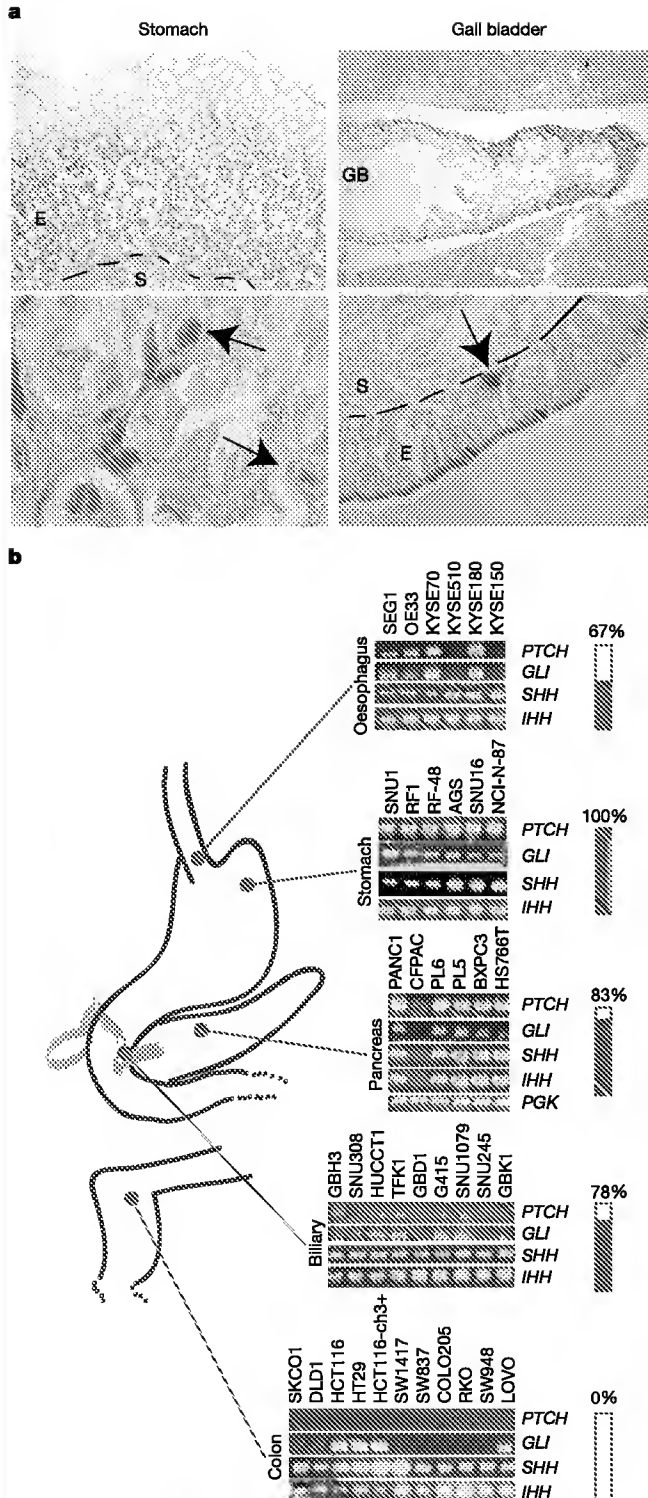


Figure 1 Hh pathway activity in normal and neoplastic gut cells and tissues. **a**, Intra-epithelial Hh pathway activation in mature murine gut tissues. Staining for β -galactosidase activity (blue) from the *Ptch-lacZ* gene reveals expression of the Hh target *Ptch* in epithelium (E) of the stomach (arrows indicate apparent parietal cells) and gall bladder (GB; arrow indicates basally oriented cell). Staining within stromal cells (S) was also seen. **b**, Widespread expression of transcripts encoding Hh pathway components in digestive tract tumour cell lines. RT-PCR products showing expression of genes encoding Hh pathway ligands (*SHH* and *IHH*) and target genes (*PTCH* and *GLI*) in cell lines taken from sites shown in the diagram on the left. Red bars indicate the percentage of lines expressing detectable *PTCH* mRNA at each site.

early endoderm and throughout gut development^{10,12}. We detected *SHH* and *IHH* messenger RNA in 37 of 38 (97%) cell lines from oesophageal, stomach, biliary tract, pancreatic and colon carcinomas (Fig. 1b). The Hh target genes *PTCH* and *GLI* were used as indicators of Hh pathway activity and were co-expressed in most cell lines from oesophageal (4/6), stomach (6/6), pancreatic (5/6) and biliary tract (5/9) tumours. By contrast, *PTCH* was not expressed in colon tumour cell lines (0/11), although a few of these cell lines expressed *GLI* in the absence of *PTCH*. The Hh pathway, however, is not active in these colon-tumour-derived cell lines, as indicated by our reporter assays (see below).

Expression of *PTCH* and *GLI* within cells from several types of digestive tract tumours that also express Hh ligands suggests the autonomous operation of an active signalling process. Autonomous pathway activity was confirmed by the high level of luciferase activity produced by an exogenously introduced Hh-inducible GLI-luciferase reporter¹³ in all cell lines producing detectable

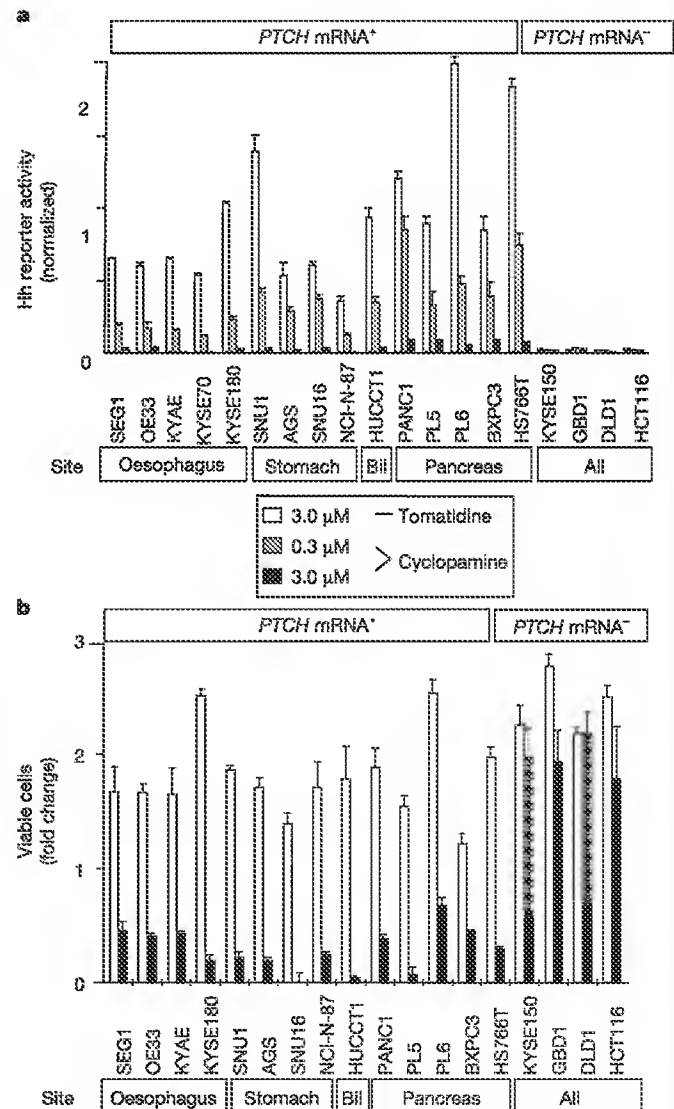


Figure 2 Cyclopamine suppression of Hh pathway activity and growth in digestive tract tumour cell lines correlates with expression of *PTCH* mRNA. **a**, Normalized activity of transiently transfected Hh-responsive luciferase reporter and dose-dependent suppression by the Hh pathway antagonist cyclopamine. **b**, Change in tumour cell viability measured by MTS (soluble tetrazolium salt) assay after culture in 3.0 μ M cyclopamine or tomatidine (control). Bili, biliary tract. Note that the viability of *PTCH* mRNA-negative cells is not affected by cyclopamine.

PTCH mRNA (Fig. 2a). Furthermore, Hh pathway activity in these cell lines was inhibited in a dose-dependent manner by the Hh-pathway-specific antagonist cyclopamine, but not by tomatidine, an inactive but structurally related compound¹⁴ (Fig. 2a). These results suggest that high levels of Hh pathway activity are a common feature of digestive tract tumours and prompted us to further investigate the role of the pathway in tumour growth. We found that cyclopamine treatment reduced the growth of tumour cell lines from the oesophagus, stomach, biliary tract and pancreas by 75–95% compared with tomatidine controls (Fig. 2b). Strikingly, significant growth inhibition was observed only in tumour lines expressing *PTCH* mRNA, confirming that the effects of cyclopamine treatment are pathway specific rather than generally cytotoxic.

Because the properties of cell lines adapted to long-term growth *in vitro* can differ from those of tumours growing *in vivo*, we also examined pathway activation in freshly resected stomach and pancreatic tumours by measuring endogenous *PTCH* mRNA levels. For each specimen, RNA for quantitative polymerase chain reaction with reverse transcription (RT-PCR) analysis was isolated from 10 consecutive 10 μ m cryosections after histological analysis of the immediately flanking sections to determine tumour content. We found that *PTCH* mRNA levels were 23–271 times (mean = 129; $n = 9$) higher in stomach tumours and 69–5,044 times (mean = 448; $n = 15$) higher in pancreatic tumours than in adjacent normal tissue (Fig. 3a).

To examine the role of Hh pathway activity in growth, we analysed pancreatic carcinomas that had been passaged once as xenografts in nude mice then cultured and immediately assayed *in vitro*. Of six such xenografts, four expressed *PTCH* mRNA (data

not shown); two of these were a matched pair of primary and metastatic tumours from a single patient. All four of these *PTCH*-expressing primary xenografts expressed the GLI-luciferase reporter in a cyclopamine-sensitive manner (Fig. 3b). Cyclopamine treatment of these *PTCH* mRNA-expressing xenografts also resulted in decreased viable cell mass (Fig. 3c), demonstrating more extreme cell-killing effects of Hh pathway blockade than those observed in established tumour cell lines (Fig. 2b). By contrast, single-passage xenografts lacking *PTCH* mRNA grew equally well in control and cyclopamine-containing media (Fig. 3c), again confirming that cyclopamine effects are pathway specific rather than generally cytotoxic.

To examine the effects of cyclopamine treatment *in vivo*, subcutaneous xenografts from HUCCT1 cells, a metastatic cholangiocarcinoma cell line, were established in athymic mice. After the tumours had grown to an average size of 180 mm³, mice bearing these tumours were injected daily with cyclopamine or vehicle, and control tumours continued to grow until animals were euthanized at 22 days with an average tumour volume exceeding 800 mm³ (Fig. 3d, e). Tumours in cyclopamine-treated animals, by contrast, regressed completely by 12 days. Treatment continued for a further 10 days, followed by an observation period of 98 days. Remarkably, all treated tumours were grossly and histologically undetectable at the end of the 3 month observation period, indicating a complete and durable tumoricidal effect of blocking the Hh pathway with cyclopamine (Fig. 3d, e). As previously reported for shorter treatment courses^{7,15}, all mice survived cyclopamine treatment and the observation period with no obvious adverse effects.

These findings establish that the Hh pathway is widely activated

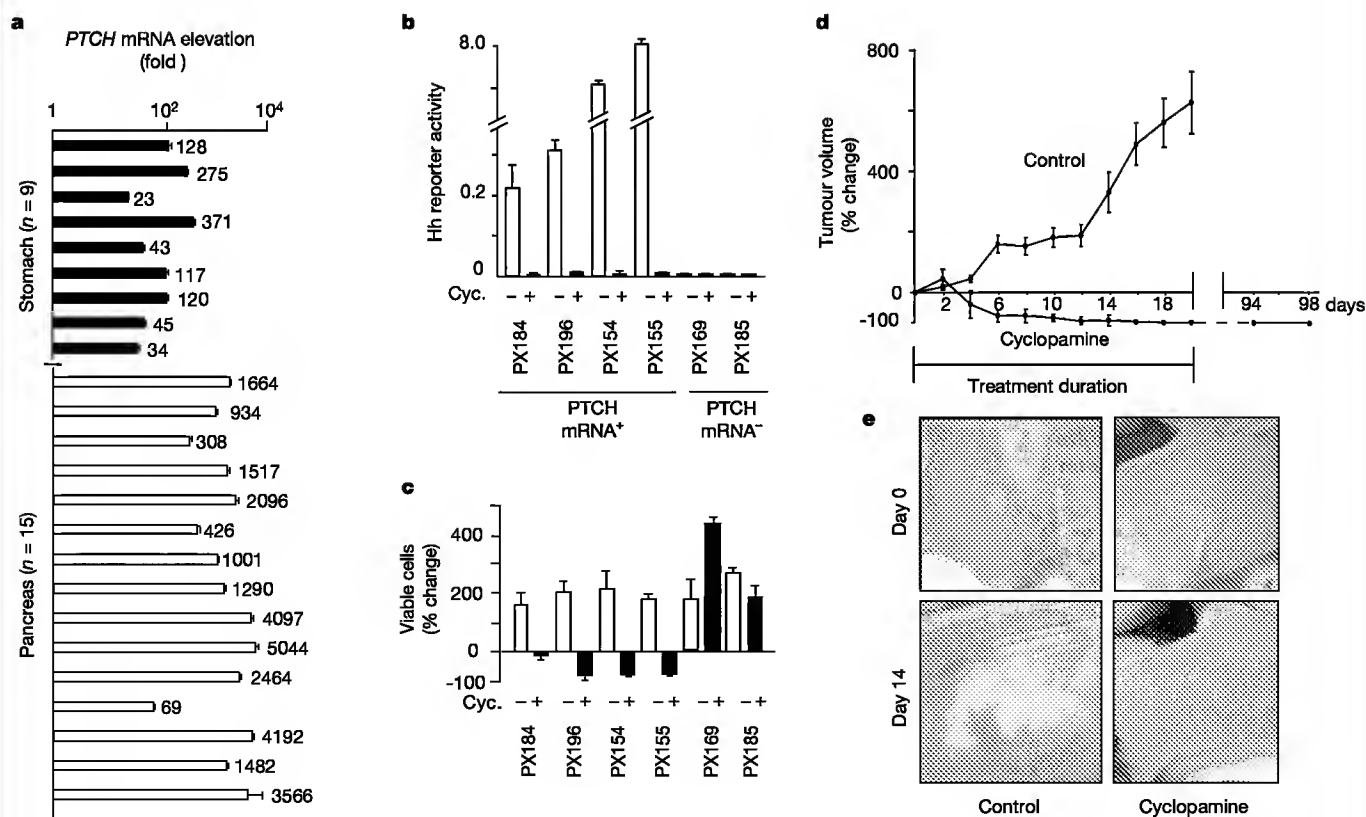


Figure 3 Hh pathway activity and requirement for growth of tumour cells *in vivo*. **a**, Elevated *PTCH* mRNA in surgically resected pancreatic and gastric carcinomas was detected by quantitative RT-PCR and normalized to adjacent normal stomach ($n = 10$) and pancreas ($n = 1$) levels. **b**, Normalized Hh-responsive reporter activity and suppression by 3.0 μ M cyclopamine in first-passage pancreas carcinoma xenografts. **c**, Corresponding reduction in viable tumour cells when cultured with 3.0 μ M

cyclopamine. Note that reduced viability is observed exclusively in lines with elevated Hh pathway activity. **d**, Change in HUCCT1 human cholangiocarcinoma xenograft volume in mice treated for 22 days with vehicle (control; $n = 9$) or cyclopamine ($n = 9$). A durable response was observed for the full 98-day observation period after cessation of therapy. **e**, Representative photographs of control and cyclopamine-treated mice. Note the full regression of the tumour on the lower right.

in gut-derived tumours, and further demonstrate a role for pathway activity in tumour cell growth *in vitro* and *in vivo*. Yet Gorlin's syndrome is not associated with a higher incidence of gut-derived tumours, and *PTCH* mutations in these tumours have not been reported, which suggests a distinct mechanism for Hh pathway activation that does not involve mutation of pathway components. Given that *SHH* and *IHH* mRNA are expressed in nearly all gut-derived tumours examined, we investigated the role of Hh ligand binding in pathway activity. We measured Hh-inducible reporter activity in cells from tumours of the oesophagus, stomach, pancreas

and biliary tract treated with 5E1 monoclonal antibody¹⁶, which binds to Shh and Ihh ligands¹⁷ and blocks signalling by disrupting ligand binding to Ptch¹⁸. Autonomous activation of the transfected reporter was not affected by the control antibody, but was markedly reduced by incubation with 5E1 at 0.1 or 10 $\mu\text{g ml}^{-1}$ (Fig. 4a and Supplementary Fig. 1a). By contrast, reporter activity was augmented by around eightfold by the addition of purified Shh ligand to a concentration of 25 nM. Addition of 5E1 in combination with Shh ligand reduced reporter activity to a level intermediate between those produced with either reagent alone (Fig. 4a and Supplemen-

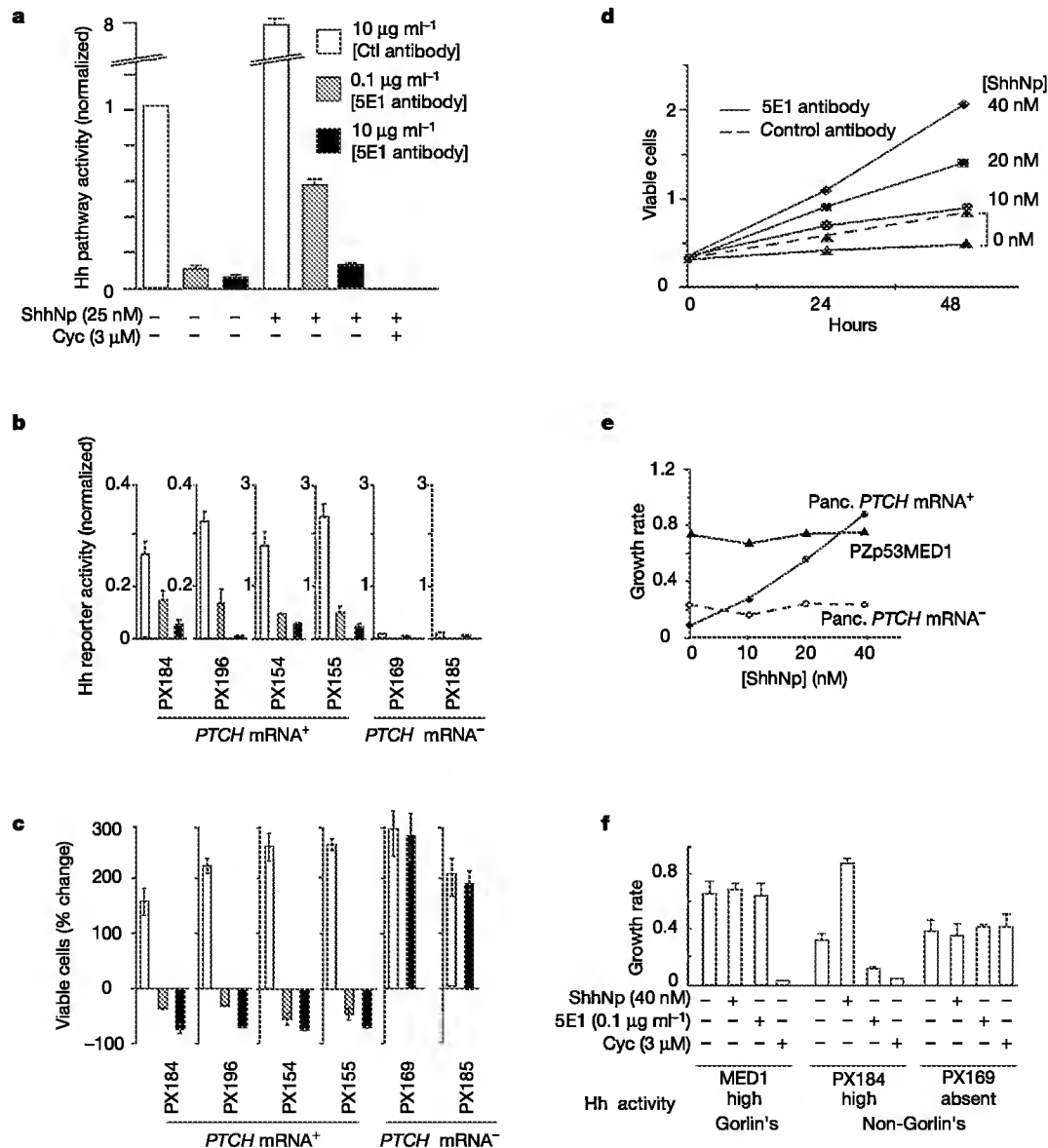


Figure 4 Ligand dependence of Hh pathway activity and growth in digestive tract tumours. **a**, Mutually antagonistic effects of the Hh ligand and blocking antibody on activity of the Hh reporter. The Hh-neutralizing 5E1 monoclonal antibody suppresses and Shh ligand increases reporter activity in HUCCT1 cells. Combined addition of antibody and ligand produces intermediate effects, depending on their relative concentrations. **b**, Hh reporter activity in first-passage pancreatic carcinoma xenografts and dose-dependent suppression with 5E1 monoclonal antibody. **c**, MTS assay showing reduced viability corresponding to Hh pathway suppression by 5E1 monoclonal antibody. **d**, MTS assay showing growth (in arbitrary units) of PX184 first-passage *PTCH*-mRNA-expressing pancreas xenograft cells cultured with the control antibody (dashed line) or with 5E1 monoclonal antibody at a level just sufficient to suppress growth (0.1 $\mu\text{g ml}^{-1}$; solid lines)

and with the indicated concentrations of added Shh ligand. **e**, **f**, Modulation of cell growth rate (in arbitrary units) by 5E1 antibody, Shh ligand and cyclopamine in single-passage pancreatic xenografts and in medulloblastoma cells (PZp53^{MED1}). **e**, Shh ligand concentrations control growth rates of *PTCH* mRNA-positive pancreatic xenograft lines (average; $n = 4$) but not of *PTCH* mRNA-negative lines ($n = 2$) or of PZp53^{MED1} cells. **f**, Opposite responses to ligand and antibody of PX-184 cells, which express *PTCH* mRNA, and the lack of response of PX-169 and PZp53^{MED1} cells, which respectively lack detectable Hh pathway activation or display constitutive pathway activation owing to lack of functional *PTCH*¹⁵. Cyclopamine, by contrast, blocks growth in cells with pathway activation because of either *PTCH* mutation or ligand stimulation.

tary Fig. 1a), which indicates a mutual antagonism between 5E1 antibody and Hh ligand in activating the pathway.

Reporter activity in cells from single-passage pancreatic cancer xenografts was also antagonized by 5E1 (Fig. 4b). In addition, treatment with the 5E1 antibody markedly reduced viable cell mass (Fig. 4c). This cell-killing effect and the reporter effect were observed exclusively in cells from tumours that expressed endogenous *PTCH* mRNA. We further investigated the relationship between ligand concentration and growth by adding 5E1 antibody to cells from a single-passage pancreatic tumour xenograft at a level that was just sufficient to block growth. We then added Shh protein and found that growth correlated positively with increasing concentrations (Fig. 4d). Rates of growth from this experiment plotted as a function of Shh concentration (Fig. 4e) indicate that ligand-induced pathway activation is rate limiting and that unperturbed growth of these cells is sub-maximal. Assays of tumour cell lines derived from the oesophagus, stomach, pancreas and biliary tract yielded similar results (Supplementary Fig. 1a–c), confirming a widespread requirement for Hh ligand in the growth of these tumours.

The Hh ligand and 5E1 antibody are mutually antagonistic in their effects on reporter activity and produce opposite effects on the growth of cells from these gut-derived tumours (Fig. 4a–e and Supplementary Fig. 1a–c). Thus, pathway activation and cell growth must be dependent on Hh ligand. By contrast, addition of neither Hh ligand nor 5E1 blocking antibody significantly affected the growth of cells from a single-passage pancreatic tumour xenograft that did not express *PTCH* mRNA (Fig. 4f), which demonstrates the specificity of antibody and ligand effects. We also observed no significant ligand- or antibody-induced change in growth of medulloblastoma cells derived from a mouse model of Gorlin's syndrome (Fig. 4f) in which the Hh pathway is activated through loss of *Ptch* function^{15,19}. In contrast to antibody-resistant xenograft cells, medulloblastoma-derived cells require pathway activity for growth and can be killed by cyclopamine treatment¹⁵ (Fig. 4f).

Ligand-independent mutational activation of the Hh pathway has been linked to the formation of tumours, such as medulloblastoma, associated with Gorlin's syndrome. Despite a widespread activation of and dependence on the Hh pathway for medulloblastoma growth¹⁵, only a fraction of sporadic tumours can be assigned to pathway-activating mutations, suggesting that other mechanisms of pathway activation may be at play. Here we establish such a mechanism by showing that pathway activation and growth of cells from a group of commonly lethal gut-derived malignancies is ligand dependent. Small-cell lung cancer, also arising from endodermally derived epithelium and associated with Hh ligand expression, has recently been linked to transient reactivation of the Hh pathway within the airway epithelium, where it regulates progenitor cell fates during injury repair⁷. A similar role for Hh signalling in renewal of mature digestive tract epithelium is suggested by expression of the Hh pathway targets *Ptch* and *Gli3*^{9,10} (Fig. 1a) and by the requirement for Hh signalling for proliferation of gut progenitor cells¹⁰. It is not known whether renewal of injured gut epithelium is associated with transient Hh pathway reactivation. However, increased rates of oesophageal, gastric and pancreatic carcinomas occur in association with acid injury in Barrett's oesophagus, in *Helicobacter pylori* infection, and with exposure to alcohol, cigarette smoke and certain dietary components^{20–22}. Exposure to such factors probably causes injury to the gut epithelium, eliciting a chronic state of injury repair and a consequent increase in proliferative stem or progenitor cells that may arise through ligand-dependent reactivation of the Hh pathway. Many of these agents are also mutagenic, thus potentially enhancing tumour formation by subjecting an enlarged pool of stem or stem-like target cells to potentially oncogenic mutations. Our results identify a group of common and frequently lethal gut-derived tumours, readily diagnosed by their expression of endogenous pathway targets such as *PTCH*, which may respond to antagonist- or

antibody-mediated pathway blockade, even at advanced stages of metastatic disease. □

Methods

Detection of β -galactosidase expression

Ptch-lacZ mice were killed at 4–6 weeks of age. Staining was performed as described previously⁷.

Tumour cells and tissues

Origins and sources of our cells and tissues are described in the Supplementary Table. First-passage pancreatic cancer xenografts were derived from freshly harvested pancreaticoduodenectomy specimens as described previously¹⁵. In previous experiments, approximately 65% of specimens yielded xenografts (data not shown), so we inferred that these xenografts represent pancreatic tumours in the general population. The diagnosis of frozen samples from gastric and pancreatic adenocarcinoma resections and adjacent normal stomach and pancreas was microscopically confirmed by two pathologists (D.M.B. and A.M.), and RNA was prepared as described previously¹⁵.

RT-PCR

Templates were prepared and amplified as described elsewhere¹⁵. For all primer pairs, specificity was confirmed by sequencing of PCR products. For quantitative RT-PCR, 10% of the first-strand reaction was amplified using IQ-SYBR Green Supermix, an i-cyclerIQ real-time detection system (Bio-Rad) and specific oligonucleotide primers for *PTCH* or *PGK* (phosphoglycerate kinase). Amplification was performed at 95 °C for 5 min followed by 40 cycles of 10, 15 and 30 s at 95 °C, 55 °C and 75 °C, respectively. Bio-Rad software was used to calculate threshold cycle (C_T) values for *PTCH* and for the housekeeping gene *PGK*. For each sample, *PTCH* expression was derived from the ratio of *PTCH* to *PGK* levels using the formula $2^{-\Delta C_T}$, where $\Delta C_T = C_{T-PTCH} - C_{T-PGK}$. *PTCH* levels in tumours were presented as a ratio to levels detected in adjacent normal tissue (Fig. 3a).

Hh-responsive reporter assays

Hh-responsive firefly luciferase and control SV40 Renilla luciferase reporter assays were performed on subconfluent triplicate cultures as described previously²³. Two days after transfection, culture medium was replaced for a 2-day culture period with assay medium: RPMI-1640 (Bio-Whittaker) supplemented with 0.5% (established cell lines) or 20% (first-passage xenografts) FBS and containing combinations of 5E1 anti-Hh monoclonal antibody, recombinant doubly lipid-modified Sonic hedgehog (ShhNp) peptide¹³, cyclopamine purified from *Veratrum* extract or tomatidine (ICN Pharmaceuticals) at the concentrations indicated in the main text. Lysates were prepared and analysed as described elsewhere¹³.

Proliferation assays

Cells were cultured in triplicate in 96-well plates in assay media to which 5E1 monoclonal antibody, ShhNp and/or cyclopamine were added at 0 h at concentrations indicated in the main text. Viable cell mass was determined by optical density measurements at 490 nm (OD_{490}) at 2 and 4 days using the CellTiter96 (Promega) colorimetric assay. Relative growth was calculated as $OD(\text{day } 4) - OD(\text{day } 2)/OD(\text{day } 2)$.

Xenograft treatment

In accordance with approved Johns Hopkins University Animal Care and Use Committee protocols, HUCCT1 tumours ($n = 18$) were grown in athymic (nude) mice and treated with cyclopamine ($50 \text{ mg kg}^{-1} \text{ d}^{-1}$, subcutaneous injection) or control vehicle as described previously¹⁵.

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Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis

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Hedgehog signalling—an essential pathway during embryonic pancreatic development, the misregulation of which has been implicated in several forms of cancer—may also be an important mediator in human pancreatic carcinoma^{1–8}. Here we report that sonic hedgehog, a secreted hedgehog ligand, is abnormally expressed in pancreatic adenocarcinoma and its precursor lesions: pancreatic intraepithelial neoplasia (PanIN). Pancreata of Pdx–Shh mice (in which Shh is misexpressed in the pancreatic endoderm) develop abnormal tubular structures, a phenocopy of human PanIN-1 and -2. Moreover, these PanIN-like lesions also contain mutations in K-ras and overexpress HER-2/*neu*, which are genetic mutations found early in the progression of human pancreatic cancer. Furthermore, hedgehog signalling remains active in cell lines established from primary and metastatic pancreatic adenocarcinomas. Notably, inhibition of hedgehog

signalling by cyclopamine induced apoptosis and blocked proliferation in a subset of the pancreatic cancer cell lines both *in vitro* and *in vivo*. These data suggest that this pathway may have an early and critical role in the genesis of this cancer, and that maintenance of hedgehog signalling is important for aberrant proliferation and tumorigenesis.

Sonic hedgehog (SHH) is misexpressed in human adenocarcinoma and its precursor lesions. SHH expression was determined using *in situ* hybridization to detect SHH messenger RNA and immunohistochemistry (IHC) to detect the protein with an antibody directed against SHH⁹. Pancreatic tissues were obtained from 20 specimens resected for pancreatic cancer. Control pancreatic tissues with no evidence of abnormality or autolysis upon histological evaluation were obtained from autopsy specimens or from pancreatic resections for trauma. In normal adult human pancreata, no SHH was detected in the islets, acini or ductal epithelium (Fig. 1a). However, evaluation of pancreata from patients with adenocarcinoma reveals that SHH is aberrantly expressed in 70% of specimens. Normal ductal epithelium does not express detectable levels of SHH (Fig. 1b); however, as the ductal epithelium shows increasing degrees of atypia, PanIN-1 to -3 (Fig. 1c–e), a higher expression of SHH is observed. SHH expression is also detected in the malignant epithelium of adenocarcinoma samples (Fig. 1f). This expression pattern was also confirmed by our *in situ* hybridization for SHH mRNA (Supplementary Fig. 1).

Loss of regulation in this pathway has been implicated in several human cancers^{10,11}. Thus in order to determine the potential role of SHH misexpression in the adult human pancreas, pancreata from

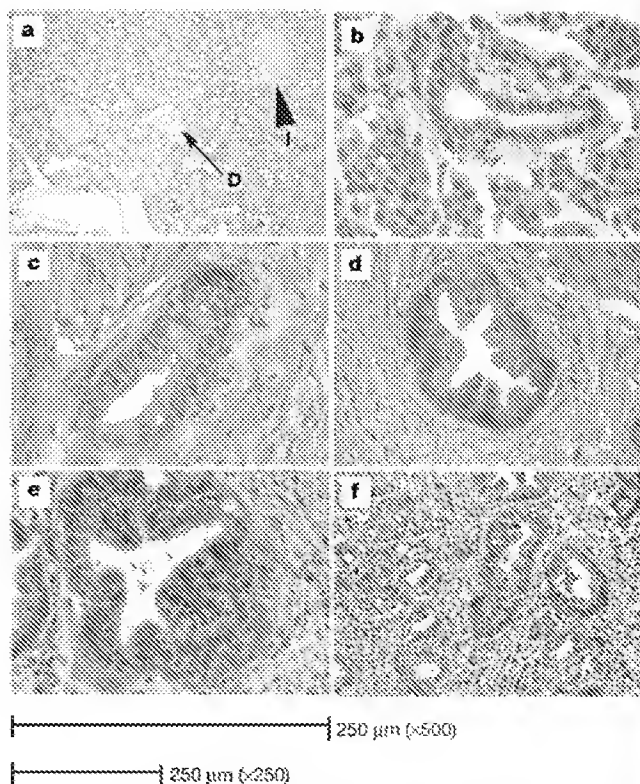


Figure 1 Immunohistochemical identification of SHH. **a**, Normal human pancreas. No specific staining for SHH protein was identified in acini, islets (I) or ducts (D) ($\times 125$ magnification). **b**, No SHH expression is detected in normal ductal epithelium ($\times 500$ magnification). **c**, PanIN-1 expresses minimal amounts of SHH ($\times 500$). **d**, PanIN-2 expresses moderate levels of SHH ($\times 250$). **e**, PanIN-3 ($\times 250$) and **f**, Invasive cancer ($\times 125$): moderate to high levels of SHH.